Do Green and Golden Bell Frogs *Litoria aurea* occupy habitats with fungicidal properties?

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ABSTRACT

The Green and Golden Bell frog *Litoria aurea* is in major decline in Australia, where its distribution is now confined mainly to the east coast of New South Wales (NSW). Infection by the newly emerged amphibian fungal pathogen *Batrachochytrium dendrobatidis* has been identified as one of the main threats affecting *L. aurea*. Surprisingly, some of the sites in NSW sustaining the largest populations of this species are industrial and urban habitats that are often disturbed and polluted, which could protect *L. aurea* from chytrid infection if pollution had fungicidal capacity. The aim of this study was to characterise the trace metal concentration of several *L. aurea* breeding sites in the Sydney and Illawarra regions of NSW and to evaluate the fungicidal efficacy of the main trace metals identified. Selected *L. aurea* sites were sampled throughout the breeding season (September to February) to establish the concentration of trace metals in both surface sediment and waters. Physico-chemical parameters including pH and salinity were also measured. Of the trace metals identified, copper and zinc were consistently elevated across sites. Over 50% of sites exceeded the National Sediment Quality Guideline for both copper and zinc concentration, and over 90% of sites exceeded the National Water Quality Guideline for these metals. Consequently, we evaluated their effect on the growth and survival of a laboratory culture of *B. dendrobatidis*. These tests were performed in media containing dissolved metal concentrations of 0.02 - 0.65 mgL^{-1} Cu and 0.24 - 5.0 mgL^{-1} Zn. Growth rates were inferred by total fungal density in liquid culture (based on spectral absorbance measurements), final dry weight, and the density of zoospores in fungal cultures grown for 28 days. Both copper and zinc were found to reduce the growth and proliferation of *B. dendrobatidis*, but in a non-linear manner. This suggests that *L. aurea* may be gaining some protection from *B. dendrobatidis* infection at several of the sites examined.

Key words: *Litoria aurea*, chytrid, *Batrachochytrium dendrobatidis*, trace metals

Introduction

The Green and Golden Bell frog *Litoria aurea* (Lesson 1829) has undergone a rapid population decline in south-eastern Australia, where its distribution is now confined mainly to the east coast of New South Wales (NSW) (DEC 2005). Amongst the suspected threats to the survival of *L. aurea*, the novel fungal pathogen *Batrachochytrium dendrobatidis* is considered to be particularly damaging (DEC 2005). Of the known *L. aurea* populations, reports of *L. aurea* with a *B. dendrobatidis* infection have only come from NSW (Speare and Berger 2005). There have been reports of infected *L. aurea* frogs in some Sydney populations, including those found in Sydney Olympic Park (Penman *et al.* 2008). One outbreak in a Sydney population was believed to have occurred after invasion of *L. aurea* habitat by the Brown-striped Frog *Limnodynastes peronii* (A. White and G. Pyke pers. comm.), suggesting transmission of the disease through the environment. Other *L. aurea* populations discovered to have *B. dendrobatidis* infection include those found in the Southern Highland regions around Canberra (Wassens and Mullins 2001).

In NSW, *L. aurea* is most abundant at sites that are located in current or former industrial areas, and areas that can be considered highly disturbed (White and Pyke 1996; DEC 2005). The populations persisting in these areas may either be resistant to *B. dendrobatidis* infection, or be located in areas that are inhospitable to the growth of *B. dendrobatidis*. It has been suggested that many extant *L. aurea* habitats possess characteristics such as fluctuating salinity and elevated concentrations of trace metals that have been shown to adversely affect the growth of *B. dendrobatidis in vitro* (Johnson *et al.* 2003; Parris and Baud 2004). As such, extant habitats may protect *L. aurea* from *B. dendrobatidis* infection.

It is now generally accepted that the amphibian fungal pathogen *B. dendrobatidis* has contributed to the decline of many amphibian species (Berger *et al.* 1998; Bosch *et al.* 2001; Lips *et al.* 2003; Muths *et al.* 2003; Woodhams and Alford 2005). Infection with *B. dendrobatidis,* a member of the phylum Chytridiomycota (Longcore 1999), causes the cutaneous disease chytridiomycosis. *Batrachochytrium dendrobatidis* has two life stages, the zoosporangia, and the zoospores (Longcore *et al.* 1999). It is known that zoospores of *B. dendrobatidis* are water borne, however, the zoosporangia require a substrate containing protein to settle upon in order to mature and produce zoospores (Johnson and Speare 2003; Longcore *et al.* 1999). It has also been shown that *B. dendrobatidis* can grow for up to seven weeks in sterile lake water, and up to three months in sterile, moist river sand (Johnson and Speare 2003, 2005). The ability to live in both water and on a substrate suggests that *B. dendrobatidis* utilises both components within its environment.

Many natural agents can collectively alter the intensity of *B. dendrobatidis* infection in some areas (Pounds *et al.* 2005). As such, this disease can act in concert with other environmental perturbations, leaving amphibians more susceptible to infection (Pounds *et al.* 2005). However, there are various factors which could act to hinder the growth of *B. dendrobatidis* in the environment, and thereby lower the rate of infection, including temperature, pH, salinity and humidity, particularly when outside the ranges considered optimal for *B. dendrobatidis* growth (Johnson *et al.* 2003; Piotrowski *et al.* 2004).

Batrachochytrium dendrobatidis is also susceptible to a range of chemical agents including fungicides and disinfectants (Johnson *et al.* 2003; Parris and Baud 2004). Trace metals have long been known to be effective fungicidal agents (Ross 1975), although some metals essential for growth at low concentrations exhibit varying levels of toxicity above a certain concentration (Gadd 1993). As *B. dendrobatidis* has only been newly discovered, relatively few studies have looked at the effect of trace metals, or any other compounds on *B. dendrobatidis* (Johnson *et al.* 2003; Parris and Baud 2004). As such, there is a large gap in knowledge regarding the effects of trace metals on *B. dendrobatidis*, both *in vitro* and under field conditions.

The aim of this study was to investigate the fungicidal potential of *L. aurea* breeding sites in the Sydney and Illawarra regions. The physical qualities of *L. aurea* breeding habitats were assessed because it has been suggested that they may contain contaminants that afford *L. aurea* some protection from *B. dendrobatidis* infection (DEC 2005). A laboratory examination investigated the fungicidal capacity of the two main trace elements identified in *L. aurea* habitats, copper and zinc, against *B. dendrobatidis*.

Methods

Study area

Preliminary sampling was undertaken at known breeding sites of *L. aurea* in the Sydney and Illawarra regions. Priority was given to sites that had relatively large populations and which were close to potential sources of contaminants. Two locations were selected for study in the Sydney region (Table 1). These were within Sydney Olympic Park, where three separate sites were sampled, and on the Kurnell Peninsula at Towra Lagoon. Within the Illawarra region, eight sites were chosen for investigation (Table 1). Two were situated on private industrial land and the remainder were in close proximity to these industrial properties, or other nearby industries. Samples were also collected at newly formed ephemeral ponds surrounding South Pond, in Port Kembla, a breeding site undescribed before this study.

Analytical Method

All field sediment and water samples, and all laboratory water samples, were placed in 50 mL polyethylene containers which were previously washed in metal-free detergent (Decon 90, Biolab Scientific Pty Ltd), rinsed three times in de-ionised water, soaked in 10% (v/v) nitric acid for >24 h, and rinsed three times in MilliQ water (18MΩ/cm resistivity). All other plastic and glassware used were washed in the same manner. Water used for sample preparation was filtered using 0.45 μm cellulose acetate filters (Sterile Millex filter unit, Millipore), which had been washed with acid (2% v/v HNO_3). All chemicals used to prepare solutions were of analytical grade and dissolved in Milli-Q water.

Site (NSW Australia)	Last recorded sighting	Tenure	Reference
Sydney			
Sydney Olympic Park	2006^	SOPA	Dept. of Environment and Conservation 2005
Towra Point Lagoon	1977	NP	Australian Littoral Society 1978
Illawarra			
Bellambi Lagoon	2004*	LG	Dept. of Environment and Conservation 2005
Coomaditchy Lagoon	1999*	LG	Goldingay & Newell 2005
	2001		
Killalea Lagoon	1997*	State Reserve	Dept. of Environment and Conservation 2005
Korrungulla Wetland	1998*	LG	Goldingay and Lewis 1999
Industrial Site 1	1999*	Private	Goldingay and Lewis 1999
Industrial Site 2	2006^	Private	Biosphere Environmental Consultants 2001
South Pond	2006^	Crown	Goldingay and Lewis 1999
South Pond surrounding areas	2006^	Crown	-
Tom Thumb Lagoon	-	LG	McGregor, S pers comm. 2005, WCC

Table 1. Sites chosen in Sydney and the Illawarra for investigation of the Green and Golden Bell frog *Litoria aurea*. Symbols and abbreviations: ^Sighted during this study, *Species sighting listed on the DEC Wildlife Atlas – (Department of Environment and Conservation), WCC = Wollongong City Council. Tenure; SOPA = Sydney Olympic Park Authority, NP = National Park, LG = Local Government, Crown = Federally owned.

Field sampling

Sediment and water samples were collected from the sites listed in Table 1 from October through February 2005-06. Four to six replicates were taken per site depending on the size of the water body. Sediments were collected from the top 5 cm of the sediment using a polyethylene corer and placed into a 50 mL polyethylene container. Sediment pH was recorded *in situ* using a pH probe (Orion 290) that was pre-calibrated using pH 4 and 9 reference standards. Surface water samples were taken between 1- 2 m from the water's edge. Water samples were filtered on site through pre-washed 0.45 μm cellulose acetate filters into 50 mL polyethylene containers using 60 mL syringes (Terumo). Both syringes and filters were pre-rinsed with the sample before the final sample was taken, and samples were then acidified to 1% (v/v) HNO_3. Sediment and water samples were kept on ice immediately after collection and stored below 4ºC until analysis. In the laboratory, sediments were dried at 50°C for 48 h, homogenized and sent to an external laboratory for analysis (see below).

The variables pH, salinity (‰), turbidity (NTU), temperature (°C), dissolved oxygen (mg L^{-1} and % saturated) and electrical conductivity ($\mu S.cm^{-1}$) were measured using either a calibrated Yeokal (Model 611, Yeokal Electronics), or YSI (Model 6820 Multiprobe). These measurements were taken from 2-4 areas at each site in the early, mid and late breeding season (September 2005 until March 2006).

Toxicity experiment

To investigate the toxicity of copper and zinc on *B. dendrobatidis* a 28-day toxicity test was carried out. Vials containing an aliquot of the fungal culture were spiked with a known amount of metal solution and subsequent fungal growth measured.

Fungal culture and test solutions

The *B. dendrobatidis* culture used in this experiment was maintained at the University of Sydney but had been obtained from the AAH Laboratory, CSIRO Livestock Industries, Geelong, Victoria. This strain was originally isolated from an infected Lesueur's treefrog (*Litoria lesueuri*). Subcultures were incubated at 19°C in slanted falcon tubes containing liquid nutrient media (TGhL media). TGhL media was made by dissolving 16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose in 1000 mL of synthetic water. Synthetic hard water contained 192 mg L^{-1} $NaHCO_3$, 120 mg L^{-1} $CaSO_4$, 120 mg L^{-1} $MgSO_4$ and 8 mg L^{-1} KCl, which was aerated overnight to give an alkalinity of 57 to 64 (mg $CaCO_3$ L^{-1}), water hardness of 160 to 180 (mg $CaCO_3$ L^{-1}) and a pH of 7.6 to 8 (US EPA 2002). The antibiotics Penicilin G sodium salt (100 μgL^{-1}) and Streptomycin (100 μgL^{-1}) (Sigma-Aldrich, Castle Hill, Australia) were added to the liquid media to prevent bacterial contamination. The solid media used for inoculation was PmTG agar (1 g peptone, 1 g ryptone, 2.5 g glucose, 5 g agar, 500 mL deionised water). Nutrient solutions were autoclaved at 121ºC for 20 minutes before use. Fungal culture and liquid media used in toxicity tests consisted of synthetic hard water and TGhl media.

The metal concentrations chosen for toxicity testing were based on results in the current study, and were consistent with a previous study of water quality in *L. aurea* breeding sites in the Illawarra (R. Goldingay unpubl. data). Solutions of each metal were prepared using copper and zinc sulfate stock solutions, and contained TGhl media and synthetic hard water. An aliquot of each test solution was analysed by ICP-MS to determine the actual dissolved metal concentration (mg L^{-1}) prior to inoculation. Test concentrations were 0.022, 0.0385, 0.06, 0.332, 0.652 mg L^{-1} copper, and 0.236, 0.276, 0.7645, 1.875, 4.966 mg L^{-1} zinc, where the lowest concentration in each served as the control treatment. The pH of these test solutions was maintained throughout the experiment at 7.0 ± 0.5.

Inoculation and growth measurements

Before use in toxicity tests, the *B. dendrobatidis* culture used was standardised to an optical density of 0.06 by diluting the stock culture with Milli-Q. All measurements of optical density were taken using a UV/Visible Spectrophotometer (Ultrospec 2100 pro) at a wavelength of 495 nm using a 1 cm cell (Piotrowski *et al.* 2004). All toxicity tests were carried out in 50 mL glass Schott bottles. A 19 mL aliquot of each copper and zinc metal test solution was placed in the Schott bottle and inoculated with a 1 mL aliquot of standardised *B. dendrobatidis* culture. Each test concentration was replicated four times, with a fifth control replicate inoculated with 1 mL sterile Milli Q (to establish a baseline on the spectrophotometer). Absorbance measurements were taken every 48 h for 14 days, then taken again on Day 28. To examine the recolonising ability of *B. dendrobatidis* at Day 14 and Day 28, a 1 mL aliquot from each replicate was taken and plated onto PMtg agar, incubated for 48 h, then visually inspected for fungal growth and live zoospores using a microscope (Olympus Vanox). Also at Day 14 and Day 28, a 20 μL aliquot was removed from each replicate (n=4) and the density of zoospores (cells mL^{-1}) was measured using a haemocytometer (Marienfeld 0.0025mm^2). At the termination of the experiment, all fluid remaining in vials was filtered through pre-dried and weighed filter papers (55 mm Whatman filter papers), and the dry weight of the *B. dendrobatidis* remaining in the vials determined after oven drying at 60°C for 24 h.

Metal determinations

All analyses were conducted by Environmental Analysis Laboratories (EAL), Southern Cross University, NSW, which is a NATA accredited laboratory (accreditation number 14960). Sediments were analysed for acid extractable trace metals (mg/kg dry weight) by digestion with a weak acid (1M HCl), as described in Simpson *et al.* (2004). Water samples and toxicity test solutions were analysed directly for their dissolved metal concentration (μgL^{-1}). All metal concentrations were determined by Inductively Coupled Plasma–Mass Spectrometry (ICP–MS), with the exception of Fe and Al, which were quantified using Inductively Coupled Plasma–Optical Emission Spectrometry (ICP-OES). For quality control purposes, replicate samples and blanks were analysed and were found to be within agreement (<5% standard deviation).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to determine whether the optical density of trace metal treated vials and controls differed at Days 2, 6, 14 and 28. Variations in number of zoospores produced (cells mL^{-1}) after Day 14 and Day 28, and in dry weight (mg) between controls and trace metal treated samples after Day 28 were also investigated via a one-way ANOVA. Significant differences between groups were further analysed using a Tukey–Honestly Significant Difference test. Data were investigated for normality by inspection of the distribution of residuals and a Shapiro-Wilks test. Any data not normally distributed were transformed using a log (x+1) transformation.

Results

L. aurea habitat qualities

The pH range of sediment within *L. aurea* habitats varied widely, ranging from pH 5.9 at Industrial Site 2 to pH 9.1 at South Pond (Table 2). Many trace metals were also identified in the sediment of the *L. aurea* habitats (See Appendix Table A1 for all metals analysed), several exceeding the limit of the National Sediment Quality Guidelines (ANZECC and ARMCANZ 2000), with over 50% of sites exceeding the guidelines for concentrations of cadmium, copper, lead and zinc (Table 2).

Physico-chemical water parameters varied throughout the breeding season at all sites (Table 3). The site with the most acidic waters was Killalea Lagoon, with a pH of 6.01, and the most alkaline site was South Pond with a pH of 10.24. All other sites only deviated slightly from acceptable Freshwater Quality Guidelines (ANZECC and ARMCANZ 2000), at near-neutral pH values. The salinities at most sites remained fairly constant at below 2 ppt throughout the season, except at Towra Point (40 ppt), which was also the most shallow. All other variables measured deviated from acceptable values listed by the National Guidelines (ANZECC and ARMCANZ 2000), further indicating that the habitats sampled were of poor water quality. Of the trace metals identified in the waters of *L. aurea* habitat, several were consistently elevated across sites (Table 3; see Appendix 1, Table A2 for all metals analysed). In particular, 12.5% of sites exceeded the limit of the National Freshwater Quality Guidelines for lead, 94% of sites exceeded the guideline for copper, and 100% of sites exceeded the guidelines for zinc (ANZECC and ARMCANZ 2000).

Toxicity of copper and zinc to *B. dendrobatidis*

Copper had an acute inhibiting effect on fungal growth, but this inhibition diminished over the 28 days (Figure 1). The growth rate of copper-exposed *B. dendrobatidis* was very slow for the first six days, as revealed by optical density decreasing slightly between Day 0 and Day 2, after which no growth was observed until Day 6. Growth increased rapidly between Day 6 and Day 28 where the control treatment (0.02 mg L^{-1} Cu) had the highest final optical density of all treatments. However, none of the treatments differed significantly in optical density compared to the controls after Day 2, 6, 14 or 28 (all were $F_{4,15} > 0.67$, $p > 0.40$). The lack of significant differences between treatment groups was influenced by high variation among replicates, as indicated by the large standard errors (Figure 1).

There was a similar initial decrease in optical density of *B. dendrobatidis* exposed to zinc, with no growth evident until after Day 6 (Figure 2). Fungal growth was substantial after Day 6, reaching a growth plateau by Day 14 and maintained until test termination on Day 28. There was no significant difference between treatments when

Table 2.

Site	pH	Cadmium (mg/kg)	Copper (mg/kg)	Lead (mg/kg)	Zinc (mg/kg)
SOP-BrickPit	8.5 ± 0.08	0.2 ± 0.04	27.2 ± 10*	45.6 ± 13.8	127.4 ± 35.5
SOP-Narrawang	7.2 ± 0.25	0.11 ± 0.07	12.6 ± 5.1	34.5 ± 19.0	63.3 ± 36.8
SOP-NWF	7.6 ± 0.29	0.17 ± 0.04	26.2 ± 94.3	59.0 ± 25.4*	19.3 ± 15.4
Towra Point	-	0.24 ± 0.1	16.3 ± 8.9	39.3 ± 21.8	86.7 ± 43.5
Bellambi Lagoon	6.9 ± 0.0	0.14 ± 0.03	24.0 ± 2.54	12.9 ± 1.3	55.1 ± 9.8
Coomaditchy Lagoon	6.7 ± 0.06	3.99 ± 2.34*	280.0 ± 84*	171.0 ± 55.3*	791.8 ± 373.7*
Killalea Lagoon	6.3 ± 0.08	0.16 ± 0.03	91.8 ± 23.2*	15.6 ± 3.7	37.6 ± 10.1
Korrungulla Wetland	7.9 ± 0.19	0.14 ± 0.07	48.7 ± 27.1	13.23 ± 10.1	244.0 ± 219.4*
Industrial Site 1a	7.8 ± 0.02	3.26 ± 0.28*	179.0 ± 71.7*	126.9 ± 50.6*	791.6 ± 240.6*
Industrial Site 1b	7.3 ± 0.14	3.25 ± 0.54*	746.0 ± 170*	240.0 ± 40.6*	1223.0 ± 101*
Industrial Site 1c	7.6 ± 0.14	0.9 ± 0.46*	143.9 ± 77.4*	84.6 ± 33*	346.3 ± 52.4*
Industrial Site 1d	8.0 ± 0.17	1.75 ± 0.74*	480.0 ± 231.8*	183.3 ± 44.6*	1201.0 ± 270*
Industrial Site 1e	7.7 ± 0.0	3.3*	879.0*	255.3*	1622.0*
Industrial Site 2	5.9 ± 0.15	0.11 ± 0.01	72.0 ± 27.1*	20.7 ± 8.0	37.7 ± 15.3
South Pond	9.1 ± 0.95	0.55 ± 0.16*	85.8 ± 12.2*	61.1 ± 11.3*	914.6 ± 263.8*
Tom Thumb Lagoon	7.6 ± 0.2	0.9 ± 0.38*	134.0 ± 51.7*	123.2 ± 58.7*	971.2 ± 563*
ANZECC Guideline	-	0.5 - 10	65 - 270	50 - 220	200 - 410

Table 3.

Site	pH	Salinity (ppt)	Copper (μgL^{-1})	Lead (μgL^{-1})	Zinc (μgL^{-1})
SOP-BrickPit	8.6 – 9.0*	1.6	4.0 ± 1*	1.0 ± 0	35.0 ± 13*
SOP-Narrawang	7.5 – 8.5	1.7	3.0 ± 2.3*	< 0.01	10.0 ± 2.3*
SOP-NWF	7.6 – 9.1*	0.3	3.0 ± 0.42*	< 0.01	23.0 ± 0.54*
Towra Point	7.2 – 8.7*	40.0	< 0.05	1.0 ± 0.24	11.0 ± 0.8*
Bellambi Lagoon	7.1 – 8.7	0.3	3.0 ± 0.46*	< 0.01	48.0 ± 27*
Coomaditchy Lagoon	7.7 – 9.1*	0.2	7.0 ± 0.62*	1.0 ± 0.23	38.0 ± 9.8*
Killalea Lagoon	6.0 – 9.1*	0.4	2.0 ± 0.03*	< D.L	33.0 ± 9.9*
Korrungulla Wetland	7.3 – 8.9*	0.8	3.0 ± 0.07*	1.0 ± 0.03	48.0 ± 13*
Industrial Site 1b	7.4 – 8.6	0.5	5.0 ± 0.63*	1.0 ± 0.31	21.0 ± 3.7*
Industrial Site 1c	8.0 – 8.4*	0.6	3.0 ± 0.09*	8.0 ± 0.9*	15.0 ± 2.6*
Industrial Site 1e	7.5 – 8.9*	0.2	2.0 ± 1*	< D.L	26.0 ± 7*
Industrial Site 2	7.2 – 8.4	0.1	6.0 ± 0.69*	1.0 ± 0.06	33.0 ± 7.3*
South Pond	7.6 – 10.2*	0.5	89.0 ± 23*	38.0 ± 12.5*	1063.0 ± 343*
South Pond surrounding areas	8.5 – 8.8*	0.3	19.0 ± 2*	1.0 ± 0	140.0 ± 35*
Tom Thumb Lagoon	7.2 – 8.7*	0.8	3.0 ± 0.04*	< D.L	11.0 ± 0.8*
ANZECC Guideline (2000)	6.5 – 8.0	-	1.4	3.4	8

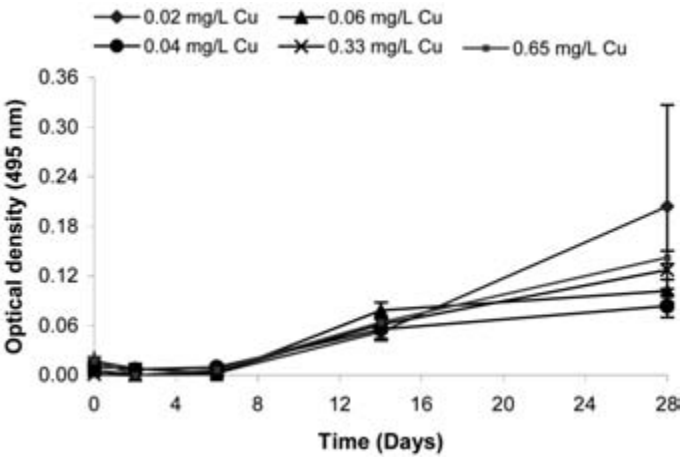

Figure 1. Optical density of *B. dendrobatidis* over a 28-day period at varying copper concentrations (mg L^{-1}). Optical density was measured at 495 nm (mean ± standard error, n = 4).

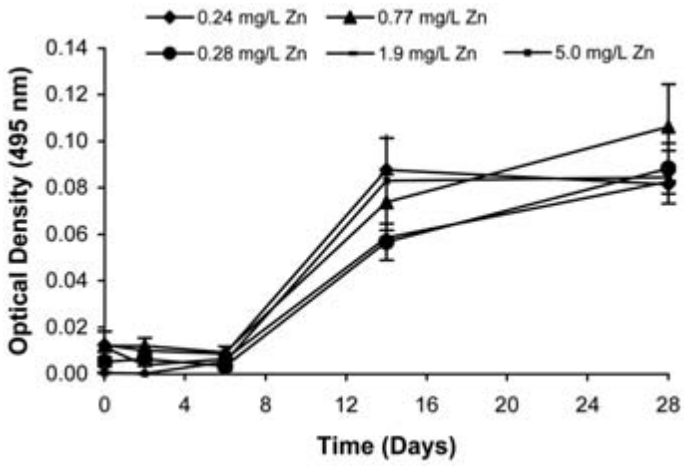

Figure 2. Optical density of *B. dendrobatidis* over a 28-day period at varying zinc concentrations (mg L^{-1}). Optical density was measured at 495 nm (mean ± standard error, n = 4).

compared to controls after Day 2, 6, 14 or 28 (all were $F_{4,15} > 0.07$, $p > 0.11$). Again, there was considerable variation among replicates, resulting in high standard errors (Figure 2). None of the fungal cultures in the zinc treatment grew as densely as those in the copper treatments.

The recolonisation of *B. dendrobatidis* was evaluated by visual inspection of agar plates that had been inoculated with a 1 mL aliquot of each treatment group. There was no effect of the copper or zinc exposure at any concentration examined on the recolonisation ability of *B. dendrobatidis* after either Day 14 or Day 28.

The dry weight of copper-treated *B. dendrobatidis* at Day 28 ranged from averages of 5.6 to 6.8 mg over the range of test concentrations (Figure 3a), however, there was no significant effect of copper concentration on fungal dry weight ($F_{4,15} = 0.986$, $p=0.445$). Following 28-days of zinc exposure, the dry weight of *B. dendrobatidis* was highly variable among concentrations. Dry weight ranged from 3.2 to 7.45 mg, where the dry weight in the 0.77 mg L^{-1} Zn treatment was significantly higher than in all other treatments ($F_{4,15} = 12.927$, $p=0.0001$; Figure 3b). This finding is consistent with the optical density measurements for these treatments measured after Day 28.

Differences were observed in zoospore density in each of the copper and zinc treatment groups only after Day 14. At Day 28, the zoospore densities in the copper treatments ranged from an average of 5.34 x 10^5 to 13.23 x 10^5 cells mL^{-1} where the difference among treatments was significant ($F_{4,15} = 7.1818$, $p=0.0019$; Figure 4a). There were significantly more zoospores produced in the second lowest copper treatment of 0.04 mg L^{-1}, than in the 0.06 mg L^{-1} and 0.33 mg L^{-1} treatments. After Day 28, the zoospore densities in the zinc treatments ranged from an average of 9.39 x 10^5 to 12.68 x 10^5 cells mL^{-1} (Figure 4b). Although there was a tendency for zoospore density to decrease as zinc concentration increased, these differences were not statistically significant.

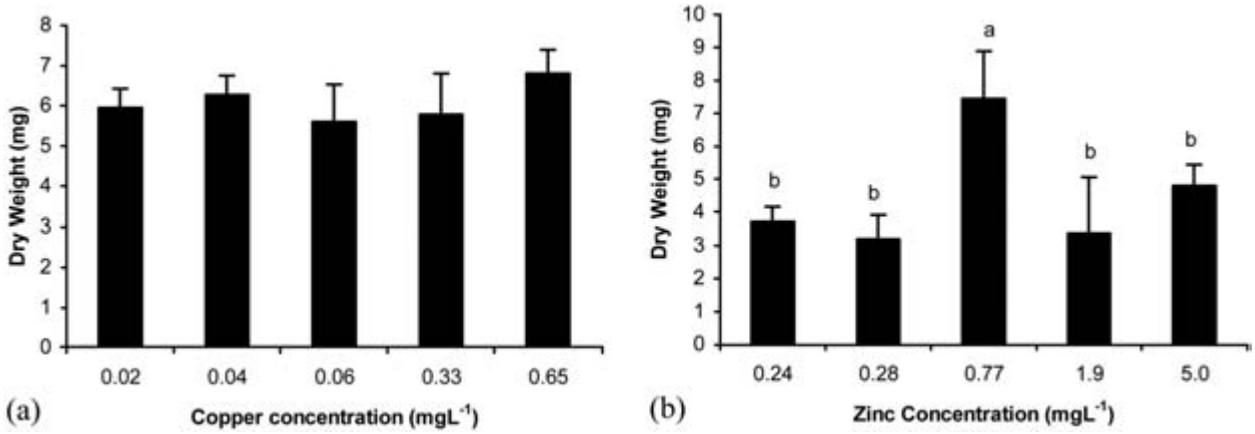

Figure 3. Dry weight (mg) of *B. dendrobatidis* after 28 days exposure to varying (a) copper concentrations (mg L^{-1}) and (b) zinc concentrations (mg L^{-1}). Results are means ± standard error (n = 4). Letters above bars denote zinc-treated concentrations that differed significantly from each other at $\alpha < 0.05$.

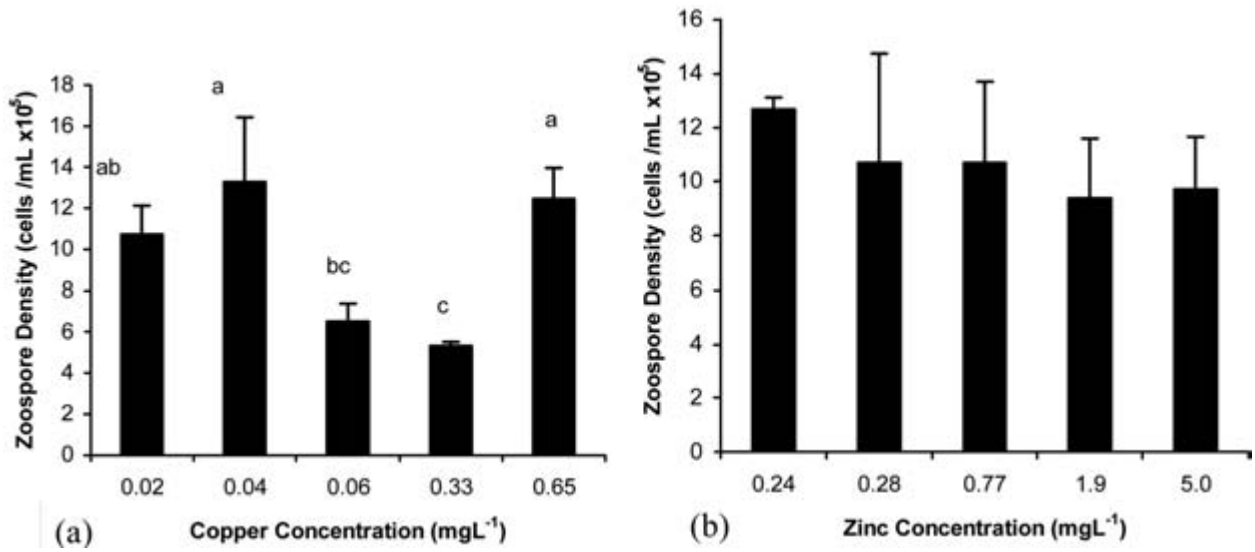

Figure 4. Zoospore density (cells x 10^5 mL^{-1}) of *B. dendrobatidis* in vitro cultures after 28 days exposure to varying (a) copper concentrations (mg L^{-1}) and (b) zinc concentrations (mg L^{-1}). Graphs represent samples taken after Day 28. Values are means ± standard error (n = 4). Cell densities were counted using a haemocytometer. Letters above bars denote copper-treated concentrations that differed significantly from each other at $\alpha < 0.05$.

Discussion

L. aurea habitat qualities

The *L. aurea* habitats most commonly used in the Sydney and Illawarra regions had physico-chemical conditions and elevated metal concentrations that should be detrimental to *B. dendrobatidis*. The optimum pH range for growing *B. dendrobatidis* is between 6 and 7 (Piotrowski *et al.* 2004). Both the sediment and water pH values in most sites fell outside this optimum range (11 of 16 sites had sediment pH in excess of 7; 14 of 15 sites had water pH in excess of 7). Most *L. aurea* sites were in close proximity to the ocean, except those in Sydney Olympic Park, and as such they are subject to either periodic tidal inundation or exposure to salt spray. Previous research by Johnson *et al.* (2003) showed that *B. dendrobatidis* does not survive exposure to high concentrations of sodium chloride. This suggests that the salinity and pH of the *L. aurea* habitats from this study could hinder the growth of *B. dendrobatidis*, restrict its distribution, and/or inhibit its ability to infect amphibians.

Previous *in vitro* studies of metal toxicity have demonstrated that copper, zinc, cadmium and lead can be fungicidal (Falih 1997; Cairney *et al.* 2001; Gharieb *et al.* 2004). Accordingly, the concentrations of these metals detected in *L. aurea* habitats have the potential to hinder the growth of *B. dendrobatidis*. What is unknown, however, is the extent to which sediment-based metals may affect *B. dendrobatidis* compared to water borne metals. There is evidence that fungi inhabiting soils are less sensitive to several trace metals than those inhabiting water (Hoiland 1994; Miersch 1997), but fungal species such as *B. dendrobatidis* exist in both sediment and aquatic environments (Johnson and Speare 2003, 2005) and would thus be susceptible to metals in both solid and liquid phases.

Effects of copper and zinc on the growth of *B. dendrobatidis*

There appeared to be an acute inhibitory effect of both copper and zinc on *B. dendrobatidis*. This was seen by the consistent decline in optical density in relation to

increasing metal concentration from inoculation at Day 0 until Day 2, where growth was inhibited by both copper and zinc treatments and did not recover until Day 6, although a similar response was measured in the control treatments that had only minimal metal concentrations present. After this time the fungus exhibited rapid growth, at rates commensurate with the controls in copper treatments, and above control values in zinc treatments. This suggests that each metal had an immediate negative effect on *B. dendrobatidis* growth, however, more measurements at shorter time intervals, and more adequate control treatments are required to validate this hypothesis.

The short-term effects of these metals on *B. dendrobatidis* growth may have occurred for several reasons. Firstly, because the concentration of free metal in solution was only measured once at the start of the experiment, there is no way to determine how much bioavailable metal was in solution as the experiment proceeded. As this was a static toxicity test, the test solutions were not renewed during the experiment, and the metal concentration in solution may have diminished over time. This phenomenon is commonly seen in short-term algal toxicity tests (Nyholm and Kallqvist 1989), and is attributed mainly to attachment of metals to glassware, adsorption to cell surfaces, and uptake and degradation by organisms (Morley *et al.* 1996; Simpson *et al.* 2003). Eventually these processes would leave the concentration of metal in solution at a much lower concentration than that provided for initial exposure.

Alternatively, any acute effect seen may have only been circumstantial. Other studies have shown that *B. dendrobatidis* zoosporangia produce motile zoospores after one to seven weeks in an aquatic media (Johnson and Speare 2003), suggesting that the initial lag period seen in optical density in this study may be a function of the long lifecycle of this fungus. As such, the fungal growth parameters measured after 14 and 28 days exposure may better represent the growth response of *B. dendrobatidis*.

The growth response of *B. dendrobatidis,* in both zoospore density and dry weight measurements, was non-linear in relation to increasing metal concentration. The results do not preclude the possibility that *B. dendrobatidis* may be susceptible to metal toxicity at both life stages, including zoospores and zoosporangia. One reason for the non-linear or distorted growth response of *B. dendrobatidis* observed may be that an excessively high density of zoospores was used initially, which resulted in a very high cell density by the end of this toxicity test. High cell density coupled with decreasing metal exposure is believed to distort growth patterns in algal toxicity tests involving metals (Nyholm and Kallqvist 1989). This combined effect could have masked any toxic effects these metals may have exhibited against the growth of *B. dendrobatidis* at more ecologically relevant concentrations.

Recommendations for future fungicidal investigations

Studies have shown that when zoospores mature to zoosporangia they become sessile, and likely settle on a substrate (Johnson and Speare 2003). Our evaluations of soil and water trace metals suggest that zoosporangia settling on substrates would be exposed to much higher metal concentrations than those we used in this experiment. We therefore recommend that future experiments investigate the combined effects of aquatic and sediment toxicity on *B. dendrobatidis*. Furthermore, these experiments should use methods assuring maintenance of a stable metal concentration throughout the experimental period, thus better resembling the large trace metal sink in the environment. If modeled on the relative concentrations of metals in soil and water, these types of experiments could verify the extent of a given habitat's fungicidal potential against *B. dendrobatidis*.

These experiments would gain further validity by varying pH and salinities in addition to varying metal compositions and concentrations, as the fungicidal efficacy of metals is known to vary with pH (Starkey 1973; Gadd and Griffiths 1980; Gibson and Mitchell 2005). The examination of the mixture of metals present in *L. aurea* environments would reveal if the metals are collectively more toxic to *B. dendrobatidis* than would be predicted from their singular effects. Synergistic effects of metals have been demonstrated to inhibit growth of other aquatic organisms (Franklin *et al.* 2002; Tsiridis *et al.* 2005).

It is also recommended that *B. dendrobatidis* be exposed separately to water and sediment collected at *L. aurea* breeding sites to see if their chemical characteristics affect fungal growth. The water and sediment should also be used in combination to further deduce pathways of toxicity to *B. dendrobatidis*. If these locations prove to ameliorate the effects of an infection, or prevent one from occurring, they would verify the importance of these sites for the conservation of the species and perhaps provide characteristics that can be used to identify alternate sites that can be used for future translocation.

Finally, to further establish the importance of sites that currently support bell frog populations, it is recommended that they be examined for the presence of *B. dendrobatidis* infection. This may involve taking skin scrapings from *L. aurea* for histological studies or for specific polymerase chain reaction-based assays, which would confirm infection in the skin of the amphibian (Berger *et al.* 1998; Annis *et al.* 2004). However, if *L. aurea* cannot be swabbed due to its scarcity, then at least common sympatric species should be assessed that may also be susceptible to *B. dendrobatidis* infection, as they may cause the spread of *B. dendrobatidis* in the area.

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APPENDIX 1

Appendix 1. Table A.1.

Table A.1. Mean Acid extractable trace metal concentration (mg/kg) in surface sediment from *L. aurea* habitat. The lower - upper National sediment quality guidelines are in bold. Values are means ± standard error, n = 4 to 6

Site	Ag	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Zn
SOP - B. Pit	0.13 ± 0.03	1939 ± 177	3.89 ± 0.54	0.2 ± 0.04	9.56 ± 0.42	27.2 ± 10.0	12400 ± 1300	0.02 ± 0	343.1 ± 39.0	10.7 ± 1.81	45.6 ± 13.8	0.69 ± 0.13	127.4 ± 35.5
SOP - Narawang	0.05 ± 0.02	1990 ± 594	1.49 ± 0.51	0.11 ± 0.07	4.83 ± 0.97	12.6 ± 5.1	7028 ± 1800	0.04 ± 0.02	149.4 ± 78	3.93 ± 1.26	34.5 ± 19.0	0.26 ± 0.02	63.3 ± 36.8
SOP - Northern Water Feature	0.08 ± 0.01	3147 ± 769	1.68 ± 0.31	0.17 ± 0.04	8.07 ± 2.12	26.2 ± 94.3	8010 ± 900	0.04 ± 0.01	110.9 ± 29	4.91 ± 0.86	59.0 ± 25.4	0.29 ± 0.03	19.3 ± 15.4
Industrial Site 2	0.08 ± 0.03	6154 ± 1395	0.67 ± 0.12	0.11 ± 0.01	8.26 ± 2.17	72 ± 27.1	13270 ± 3120	0.04 ± 0.01	166.9 ± 50.1	4.04 ± 0.95	20.7 ± 8.0	0.43 ± 0.05	37.7 ± 15.3
South Pond	0.17 ± 0.02	5771 ± 1307	2.12 ± 0.82	0.55 ± 2.34	10.1 ± 1.9	85.8 ± 12.2	8330 ± 801	0.06 ± 0.03	418.1 ± 93.5	8.15 ± 1.18	61.1 ± 11.3	0.35 ± 0.02	914.6 ± 263.8
Coomaditchy	0.38 ± 0.09	8904 ± 1580	8.56 ± 2.9	3.99 ± 2.34	17.5 ± 6.98	280 ± 84	17230 ± 3436	0.08 ± 0.02	596.5 ± 238	12.4 ± 3.22	171 ± 55.3	0.4 ± 0.02	791.8 ± 373.7
Bellambi	0.04 ± 0.01	7650 ± 2322	0.86 ± 0.13	0.14 ± 0.03	11.8 ± 1.93	24 ± 2.54	16440 ± 2985	0.02 ± 0	394.3 ± 93.6	5.53 ± 0.96	12.9 ± 1.32	0.33 ± 0.03	55.1 ± 9.8
Industrial Site 1d	0.34 ± 0.14	11168 ± 1652	4.35 ± 1.42	1.75 ± 0.74	96.6 ± 8.28	480 ± 231.8	30320 ± 2252	0.04 ± 0	3965 ± 1550	17.8 ± 2.76	183.3 ± 44.6	0.33 ± 0.04	1201 ± 270
Industrial Site 1e	0.60	10180	4.4	3.3	106	879	36388	0.04	2381	24.5	255.3	0.4	1622
Industrial Site 1b	1.23 ± 0.09	10925 ± 2291	15.82 ± 2.32	3.25 ± 0.54	95.7 ± 9.0	746 ± 170	26570 ± 7930	0.12 ± 0.03	627.6 ± 330	55.2 ± 12.2	240 ± 40.6	0.59 ± 0.07	1223 ± 101
Industrial Site 1c	0.60 ± 0.33	8271 ± 978	8.72 ± 0.83	0.9 ± 0.46	51.7 ± 12.3	143.9 ± 77.4	37495 ± 24265	0.05 ± 0.01	2990 ± 1650	30.2 ± 16.46	84.6 ± 33.0	0.52 ± 0.11	346.3 ± 52.4
Industrial Site 1a	0.62 ± 0.10	13190 ± 3953	7.52 ± 3.66	3.26 ± 0.28	129 ± 69.3	179 ± 71.7	37320 ± 11520	0.04 ± 0.01	2690 ± 1923	26.5 ± 2.02	126.9 ± 50.6	0.43 ± 0.01	791.6 ± 240.6
Killalea	0.12 ± 0.02	8397 ± 2377	0.77 ± 0.18	0.16 ± 0.03	2.23 ± 0.48	91.8 ± 23.2	7680 ± 1970	0.04 ± 0.01	132 ± 37.9	3.39 ± 0.87	15.64 ± 3.74	0.5 ± 0.07	37.6 ± 10.1
Korrungulla	0.08 ± 0.03	2507 ± 1265	0.6 ± 0.15	0.14 ± 0.07	3.24 ± 2.62	48.7 ± 27.1	4695 ± 2640	0.01 ± 0.01	176.6 ± 103	3.09 ± 1.83	13.23 ± 10.1	0.3 ± 0.05	244 ± 219.4
Tom Thumb	0.18 ± 0.05	9194 ± 514	3.25 ± 1.47	0.9 ± 0.38	31.7 ± 8.34	134 ± 51.7	21400 ± 1770	0.04 ± 0.02	699 ± 207	13.9 ± 4.78	123.2 ± 58.7	0.39 ± 0.03	971.2 ± 563.0
Towra Point	0.10 ± 0.02	1190 ± 449	2.0 ± 0.83	0.24 ± 0.1	5.1 ± 2.17	16.3 ± 8.9	2540 ± 1355	0.02 ± 0	59.7 ± 52	3.51 ± 2.19	39.3 ± 21.8	1.14 ± 0.42	86.7 ± 43.5
Sediment Guideline (mg/kg)	**1 – 3.7**	-	**20 70**	**0.5 – 10**	**80 – 370**	**65 – 270**	-	**0.15 – 1**	-	**21 – 52**	**50 – 220**	-	**200 - 410**

APPENDIX 1

Appendix 1. Table A.2.

Table A.2. Total dissolved trace metal concentration (µgL-1) in surface waters from *L. aurea* habitat. The National Freshwater quality guidelines are in bold (HF = Hardness factors, listed underneath for those elements that require it). Values are means ± standard error, n= 4 to 6

Site	Ag	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Zn
SOP - B. Pit	< 1.00	32.0 ± 7.0	5.00 ± 0.00	< 1.00	< 1.00	4.00 ± 1.00	195 ± 150	< 1.00	17.0 ± 5.0	2.00 ± 0.00	1.00 ± 0.00	8.00 ± 1.00	35 ± 13
SOP - Narawang	< 1.00	12.0 ± 6.6	2.00 ± 0.25	< 1.00	< 1.00	3.00 ± 0.23	40 ± 7.5	< 1.00	37.0 ± 4.1	1.00 ± 0.15	< 1.00	5.00 ± 0.67	10 ± 2.3
SOP - Northern Water Feature	< 1.00	84.0 ± 23.3	2.00 ± 0.24	< 1.00	< 1.00	3.00 ± 0.42	30 ± 7.2	< 1.00	53.0 ± 12.4	2.00 ± 0.30	< 1.00	1.00 ± 0.16	23. ± 5.4
Industrial Site 2	< 1.00	27.0 ± 7.5	1.00 ± 0.20	< 1.00	1.00 ± 0.15	6.00 ± 0.69	535 ± 14.2	< 1.00	167.0 ± 38.0	1.00 ± 0.12	1.00 ± 0.06	1.00 ± 0.12	33 ± 7.3
South Pond	< 1.00	3287 ± 1025	5.00 ± 0.53	1.00 ± 0.00	7.00 ± 2.07	89.00 ± 23.00	3570 ± 1190	< 1.00	402.0 ± 135	8.00 ± 1.60	38.00 ± 12.50	9.00 ± 0.88	1063 ± 343
South Pond Surroundings	< 1.00	36.0 ± 4.0	3.00 ± 0.00	< 1.00	1.00 ± 0.00	19.00 ± 2.00	102 ± 28	< 1.00	57.0 ± 20.0	2.0 ± 0.00	1.00 ± 0.00	3.00 ± 0.00	140 ± 35
Coomaditchy	< 1.00	47.0 ± 4.7	3.00 ± 0.09	< 1.00	1.00 ± 0.41	7.00 ± 0.62	160 ± 61	< 1.00	10.0 ± 1.3	1.0 ± 0.29	1.00 ± 0.23	2.00 ± 0.04	38 ± 9.8
Bellambi	< 1.00	59.0 ± 12.7	1.00 ± 0.03	< 1.00	1.00 ± 0.03	3.00 ± 0.46	92.2 ± 16.8	< 1.00	10.0 ± 1.7	1.00 ± 0.21	< 1.00	2.00 ± 0.07	48 ± 27
Industrial Site 1e	< 1.00	20.0 ± 4.0	2.00 ± 0.00	< 1.00	1.00 ± 0.00	2.00 ± 1.00	121 ± 8.0	< 1.00	1080 ± 91	1.00 ± 0.00	< 1.00	2.00 ± 0.00	26 ± 7
Industrial Site 1b	< 1.00	28.0 ± 10.9	2.00 ± 0.09	< 1.00	1.00 ± 0.08	5.00 ± 0.63	109 ± 16.3	< 1.00	279 ± 121	3.00 ± 0.07	1.00 ± 0.31	3.00 ± 0.17	21 ± 3.7
Industrial Site 1c	< 1.00	82.0 ± 7.0	4.00 ± 0.99	< 1.00	3.00 ± 0.40	3.00 ± 0.09	54 ± 7.8	< 1.00	24.0 ± 5.8	2.00 ± 0.21	8.00 ± 0.90	4.00 ± 0.33	15 ± 2.6
Killalea	< 1.00	48.0 ± 18.9	1.00 ± 0.16	< 1.00	1.00 ± 0.08	2.00 ± 0.03	153 ± 30	< 1.00	20.0 ± 5.9	1.00 ± 0.55	< 1.00	3.00 ± 0.47	33 ± 9.9
Korrungulla	< 1.00	62.0 ± 17.5	2.00 ± 0.44	1.00 ± 0.00	1.00 ± 0.16	3.00 ± 0.07	50 ± 10.3	< 1.00	55.0 ± 24.1	2.00 ± 0.38	1.00 ± 0.03	2.00 ± 0.49	48 ± 13
Tom Thumb	< 1.00	50.0 ± 21.4	2.00 ± 0.49	< 1.00	1.00 ± 0.00	3.00 ± 0.04	111 ± 35.8	< 1.00	621 ± 358	2.00 ± 0.47	< 1.00	7.00 ± 2.05	19 ± 5.2
Towra Point	2.00 ± 0.00	21.0 ± 6.5	<5.00	< 1.00	< 1.00	< 5.00	<5.00	< 1.00	7.0 ± 0.7	< 5.00	1.00 ± 0.24	<5.00	11 ± 0.8
Freshwater Quality Guideline (ugL-1)	**0.05**	**55**	**24 (iii) 13 (iv)**	**0.2 – 0.54 (HF = x 2.7)**	**1.00**	**1.4 – 3.5 (HF = x 2.5)**	**-**	**0.06**	**1900**	**11 – 27.5 (HF = x 2.5)**	**3.4 – 13.6 (HF = x 4)**	**11**	**8 – 20 (HF = x 2.5)**